Multi-pronged inhibition of airway hyper-responsiveness and inflammation by lipoxin A_4

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Published online: 12 August 2002, doi:10.1038/nm748

The prevalence of asthma continues to increase and its optimal treatment remains a challenge. Here, we investigated the actions of lipoxin A_4 (LXA_4) and its leukocyte receptor in pulmonary inflammation using a murine model of asthma. Allergen challenge initiated airway biosynthesis of LXA_4 and increased expression of its receptor. Administration of a stable analog of LXA_4 blocked both airway hyper-responsiveness and pulmonary inflammation, as shown by decreased leukocytes and mediators, including interleukin-5, interleukin-13, eotaxin, prostanoids and cysteinyl leukotrienes. Moreover, transgenic expression of human LXA_4 receptors in murine leukocytes led to significant inhibition of pulmonary inflammation and eicosanoid-initiated eosinophil tissue infiltration. Inhibition of airway hyper-responsiveness and allergic airway inflammation with a stable LXA_4 analog highlights a unique counter-regulatory profile for the LXA_4 system and its leukocyte receptor in airway responses. Moreover, our findings suggest that lipoxin and related pathways offer novel multi-pronged therapeutic approaches for human asthma.

Asthma is characterized by airway hyper-responsiveness and chronic airway inflammation[1]. Numerous eosinophils and T lymphocytes infiltrate peribronchial tissues in asthmatics[2], introducing into the lung an increased capacity to generate cysteinyl leukotrienes (CysLTs) and T_H2 cytokines[1,3,4]. CysLTs have been associated with the asthmatic diathesis in both experimental models and patients with asthma[3,5]. One of the many actions of T_H2 cytokines is to upregulate expression of biosynthetic enzymes for eicosanoids, including leukotrienes and lipoxins[7,8].

Lipoxins are a separate class of eicosanoids that are distinct in structure and function[7], and their biosynthesis is temporally dissociated from the formation and impact of other eicosanoids[9]. Lipoxins are generated in human tissues such as airways[10]. Lipoxins carry unique counter-regulatory actions that inhibit CysLT-mediated vascular responses[11] and promote resolution of cytokine-driven acute inflammation[9]. When administered to human cells *in vitro* or murine systems *in vivo*, at least two classes of receptors, CysLT1 receptors and lipoxin A_4 (LXA_4) receptors (designated ALX), can interact with lipoxins to mediate their actions[12,13]. A role for lipoxins in asthma has not yet been directly evaluated in well-qualified experimental animal models. Here, we report that a LXA$_4$ analog inhibits both airway hyper-responsiveness and inflammation in response to allergen sensitization and aerosol challenge in a murine model of asthma.

Eicosanoids in airway inflammation

After systemic sensitization to ovalbumin (OVA, 10 μg i.p.), male BALB/c mice (5–7 wk old) were exposed to aerosolized OVA (6%, 25 min) on 4 successive days. 24 hours after the last aerosol, bronchial responsiveness to intravenous (i.v.) methacholine was determined as described[14,15], bronchoalveolar lavage (BAL) was performed and tissues were collected for microscopy. Treated mice demonstrated both airway hyper-responsiveness to methacholine and inflammation (*vide infra*), including leukocytic infiltration and production of cytokines, chemokines and bioactive lipids. After allergen sensitization and aerosol challenge, we identified high levels of both CysLTs (139.0 ± 27.3 pg CysLTs per ml; mean ± s.e.m., $n = 9$) and prostaglandin E_2 (PGE_2) (1117.7 ± 103.8 pg PGE_2 per ml; mean ± s.e.m., $n = 5$) in BAL. In these same BAL fluids, LXA_4 was also present (15.0 ± 3.3 pg LXA_4 per ml, mean ± s.e.m., $n = 5$) at levels similar to leukotriene B_4 (LTB_4) (6.4 ± 2.3 pg LTB_4 per ml, mean ± s.e.m., $n = 9$), but in 10- to 100-fold lower concentrations than CysLTs and PGE_2, respectively. LXA_4 was not detected in cell-free BAL supernatants from non-immunized mice.

LXA_4 prevents airway hyper-responsiveness to methacholine

To determine if LXA_4 would protect mice from OVA-induced airway hyper-responsiveness, we administered (i.v.) 10 μg per mouse of a LXA_4 analog (LXa) that resists metabolic inactivation and blocks neutrophil accumulation and inflammatory exudate formation in murine dorsal air pouches[7,16]. When given at least 60 min before OVA challenge, LXa significantly inhibited bronchoconstriction in response to methacholine in a dose-dependent manner (Fig. 1*a* and *b*). The effective dose of methacholine required to increase lung resistance to 200% of control (ED_{200}) for mice given 10 μg LXa approximated the responses of control mice that had been sensitized but not challenged with OVA (Fig.

1*b*). Neither deleterious physiological nor behavioral effects were evident in LXa-treated mice. We also determined whether LXa administration alone would promote airway hyper-reactivity *in vivo*, and found no significant differences in ED_{200} compared with control mice (Fig. 1*b*).

LXA$_4$ inhibits allergic pulmonary inflammation

In addition to dampening airway hyper-responsiveness in OVA-allergic mice, administration of LXa significantly reduced leukocyte infiltration, in particular, tissue eosinophils and lymphocytes as well as vascular injury (Fig. 2). In BAL, total leukocytes, eosinophils and lymphocytes were sharply reduced in a dose-dependent manner (Fig. 3*a*). LXa also led to decreased T_H2 cytokines interleukin-5 (IL-5) and IL-13, as well as eotaxin in BAL fluids from OVA-sensitized and challenged animals (Fig. 3*b*). Inhibition seemed to be selective, as levels of IL-12 and tumor necrosis factor-α (TNF-α) were not similarly reduced when determined in the same samples of BAL fluid (Fig. 3*b*). LXa also regulated levels of the lipid mediators, as both PGE_2 and CysLT, but not LTB_4 were decreased in these mice (Fig. 3*c* and *d*). These results indicate that administration of LXA$_4$ mimetics can significantly inhibit features of allergic pulmonary inflammation, including leukocyte infiltration and formation of specific key mediators in airway pathophysiology.

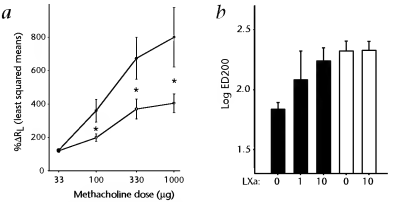

Fig. 1 Inhibition of airway hyper-responsiveness with LXa. ***a*** and ***b***, OVA-sensitized mice were treated with LXa (0–10 μg) (○) or vehicle (●) before OVA aerosol challenge. Airway reactivity was determined by methacholine-dependent change in lung resistance (*a*) and calculation of ED_{200} (*b*). In *b*, ■, OVA aerosol; □, PBS aerosol. Results are expressed as mean ± s.e.m.; $n \geq 17$ (*a*) and $n \geq 6$ (*b*); *, $P < 0.05$ by Student's *t*-test versus controls.

Human LXA$_4$ receptors dampen pulmonary inflammation

Allergen sensitization and challenge with OVA increased LXA$_4$ receptor (ALX) expression in infiltrating leukocytes and airway epithelial cells, as observed with *in situ* hybridization (Fig. 4). To assess whether OVA allergic pulmonary inflammation and airway reactivity could be regulated by ALX, we assessed the responses of transgenic mice expressing human ALX using a component of the CD11b promoter (P.R.D. *et al.*, unpublished data). After OVA sensitization and aerosol challenge, human ALX transgenic animals (ALX-tg) displayed reduced airway and vascular injury, when compared with their age and gender-matched littermate controls (non-tg). A decrease in leukocytic infiltrates occurred in peribronchial and perivascular spaces (Fig. 5*a*). BAL from allergen-challenged ALX-tg mice also had reduced numbers of total leukocytes (63% inhibition), eosinophils (68% inhibition) and lymphocytes (85% inhibition) (Fig. 5*b*). Levels of several pro-inflammatory peptide and lipid mediators were also decreased in ALX-tg mice, including IL-13 (77% inhibition), IL-5 (71% inhibition) and CysLT (74% inhibition) (Fig. 5*c*). In addition, allergen sensitization in the ALX-tg mice was blunted, as monitored by total serum IgE levels (Fig. 5*d*). Despite inhibition of allergic inflammation in the OVA-sensitized and challenged ALX-tg mice, significant differences in airway hyper-responsiveness were not observed (Fig. 5*e*) when compared with age and gender-matched littermates (peak Penh was 8.37 ± 1.89 (ALX-tg) and 8.25 ± 1.90 (non-tg), mean ± s.e. for $n = 6$).

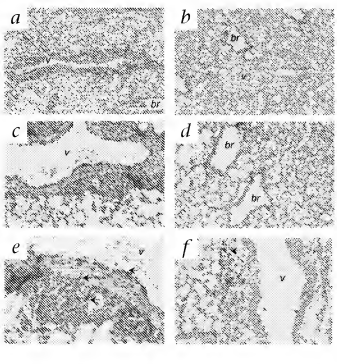

Fig. 2 Lung histopathology from LXa-treated mice. Mice were sensitized and aerosol challenged with OVA in the absence (left) or presence (right) of LXa (10 μg). Representative ($n = 3$) lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue, prepared and stained with H&E. Arrows denote eosinophils; br, bronchus; v, vessel. Magnification, ×40 (*a* and *b*), ×100 (*d*), ×200 (*c* and *f*) and ×400 (*e*).

LXA$_4$ receptors inhibit eosinophil tissue infiltration

With levels of both tissue leukocytes and pro-inflammatory mediators reduced in allergic ALX-tg mouse lungs (Fig. 5), we next examined the direct impact of human ALX expression on murine eosinophil recruitment *in vivo*. After topical application of LTB_4 (1 μg) and PGE_2 (1 μg) to mouse-ear skin, there was a significant increase in eosinophil tissue infiltration as quantified by eosinophil peroxidase (EPO) activity present within 6-mm skin punch biopsies (Fig. 6). Compared with littermate non-tg controls, expression of human ALX markedly reduced the number of eosinophils recruited in ear skin tissues from two separate murine transgenic lines (Fig. 6). Endogenous LXA$_4$ was recovered from inflamed ears and significantly different levels between ALX-tg and non-tg animals were not observed (16.0 ± 1.5 and

11.5 ± 1.9 pg/mg protein, respectively). Increased expression of human LTB_4 receptors on leukocytes (also using CD11b promoter) led to a similar amplitude of eosinophil infiltration in this model as non-tg littermate controls. Topical administration of as little as 10 μg of LXa to mouse ears inhibited eicosanoid-stimulated skin EPO activity in both non-tg mice as well as those with increased expression of LTB_4 receptors (Fig. 6). Together, these findings indicate that LXA_4–ALX interactions can mediate potent inhibition of eosinophil responses *in vivo*.

Discussion

The global prevalence of asthma continues to increase, affecting millions of peoples' daily lives, but treatment is far from ideal[17]. Clinical responses to current therapies, such as inhaled corticosteroids and leukotriene modifiers, are heterogeneous[18], and even with optimal treatment there is a substantial burden of unaddressed disease. Although the results of recent clinical trials in human asthma have challenged long-held views on the relationship between airway inflammation and bronchial hyper-responsiveness[19,20], our results indicate that LXa prevented both of these key asthma phenotypes in an experimental model of asthma; they displayed a multi-pronged impact *in vivo* that in summation seems to give beneficial airway responses.

When a longer-acting stable analog mimetic of endogenous LXA_4 was administered before OVA aerosol, airway hyper-responsiveness to methacholine as well as several measures of inflammation were markedly reduced. Pharmacological levels of LXA_4 could have direct effects on airway smooth-muscle responses to allergen challenge, as native LXA_4 given to human asthmatics inhibits LTC_4-stimulated airway hyper-responsiveness[21] and blocks LTD_4-initiated constriction of airway smooth muscle *in vitro*[22]. In addition to LXA_4's actions on its cognate high-affinity receptor (ALX), LXA_4 and the analog LXa (constructed on the aspirin-triggered 15-epimer LXA_4 biotemplate) compete for binding with high affinity at LTD_4 recognition sites and at recombinant cysLT1 receptors expressed in Chinese hamster ovary cells *in vitro*[12]. The lipoxin-mediated reductions in allergic mediators documented here could also be responsible, in part, for the observed protection from the development of airway hyper-responsiveness to methacholine. In this regard, recombinant IL-4 and IL-13 induce airway hyper-responsiveness within hours without inflammatory-cell recruitment or mucus production[23]; IL-13 decreases human airway smooth muscle β-adrenergic responsiveness *in vitro*[24]; and IL-5 and eotaxin lead to increased production of CysLTs (refs. 25,26), which are potent bronchoconstrictors[27]. Taken together, these findings suggest that endogenous lipoxins produced within the local microenvironment might temporally regulate and reduce airway hyper-responsiveness, via multiple sites of action *in vivo*, to counter-regulate key asthma-generating pathways—via inhibition of IL-5, IL-13 and CysLT-mediated actions on leukocytes, epithelia and smooth muscle.

Pulmonary eosinophilia was also sharply reduced in mice given LXa. Eosinophil recruitment to the lung in asthma is primarily a consequence of T_H2 lymphocyte activation[1], which was reduced by LXa, as shown by lower levels of T_H2 cytokines in BAL fluid and decreased number of lymphocytes in both BAL fluid and lung tissue. LXA_4 may inhibit lymphocyte recruitment by competition for ALX binding with the urokinase plasminogen-activator receptor ligand, a potent lymphocyte chemoattractant[28]. In addition, LXA_4 directly

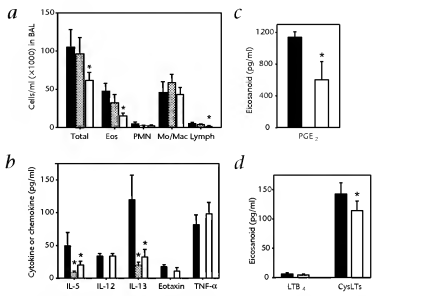

Fig. 3 LXa selectively inhibits airway leukocyte infiltration and inflammatory mediators. Bronchoalveolar lavage fluids were obtained from OVA-sensitized and challenged mice. ***a***, Leukocytes in bronchoalveolar lavage fluid were enumerated and identified after Wright–Giemsa stain. ■, control ($n = 16$); ▨, 1 μg LXa ($n = 11$); □, 10 μg LXa ($n = 21$). ***b–d***, Mediator profile of lipid mediators PGE_2 (◊), and specific indicated cytokines (○), and leukotrienes (***d***) were determined by ELISA in BAL from animals receiving LXa or vehicle as noted for *a*. Results are expressed as mean ± s.e.m. ($n \geq 6$, $d \geq 2$). *, $P < 0.05$ by Student's *t*-test versus controls.

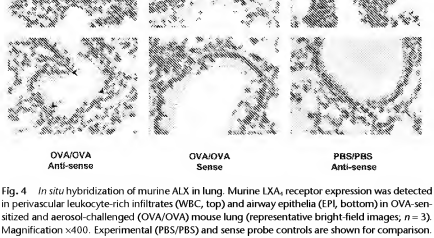

Fig. 4 *In situ* hybridization of murine ALX in lung. Murine LXA_4 receptor expression was detected in perivascular leukocyte-rich infiltrates (WBC, top) and airway epithelia (EPI, bottom) in OVA-sensitized and aerosol-challenged (OVA/OVA) mouse lung (representative bright-field images; $n = 3$). Magnification ×400. Experimental (PBS/PBS) and sense probe controls are shown for comparison. Arrows indicate expression of ALX.

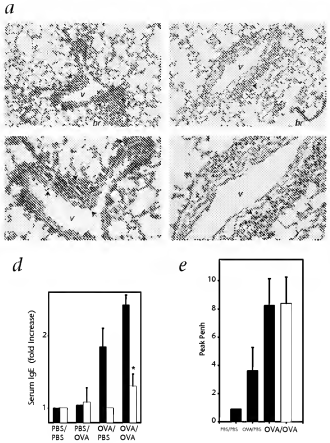

Fig. 5 Expression of human ALX in transgenic mice decreases pulmonary inflammation. ***a***, Lung histopathology from human ALX transgenic mice. Non-transgenic (left) and human ALX-tg mice (right) were sensitized and aerosol challenged with OVA. Representative ($n = 6$) lung tissue sections were obtained from formalin-fixed, paraffin-embedded lung tissue, prepared and stained with H&E. br, bronchus; v, vessel. Arrows indicate infiltrating leukocytes. Magnification, ×40 (upper) and ×200 (lower). ***c***, BAL fluids were obtained, leukocytes enumerated and identified after Wright–Giemsa stain. ■, non-transgenic; □, ALX-transgenic (for *c–e*). Percent inhibition in cell number (*b*) and changes in mediator profile of indicated cytokines and lipid mediators (*c*) were determined. Total serum IgE levels were determined by immunoassay in samples from mice sensitized or challenged with either OVA or buffer (PBS) (*d*). Airway reactivity was determined by methacholine (100 mg/ml)-dependent change in Penh (*e*). Results are expressed as mean ± s.e.m. ($n = 4$, $d \geq 2$). *, $P < 0.05$ by Student's *t*-test versus control animals.

inhibits eosinophil chemotaxis[29], and several of its stable analog mimetics block eotaxin formation *in vivo* in parasite animal models[30], promoting resolution of allergen-mediated pleural inflammation[31]. Although endogenous lipoxin production paralyzes splenic dendritic-cell responsiveness to IL-12 and IL-12 production in microbial immunity[32], LXa administration during allergen challenge here did not significantly alter IL-12 levels in BAL fluids. This finding suggests distinct sites of LXA_4 action in allergic inflammation down-stream from dendritic cells. In aggregate, our results demonstrate potent inhibition by LXa of both T_H2 lymphocyte and eosinophil recruitment *in vivo*—processes that characterize asthma pathobiology. Moreover, the capacity for LXa to inhibit these inflammatory responses raises the likelihood that endogenous lipoxin production serves as a pivotal regulatory event in airway and allergic inflammation.

Lipoxins were generated endogenously during allergen-induced airway inflammation. The amounts of LXA_4 recovered were similar to the levels of LTB_4, yet amounts of both of these eicosanoids were substantially lower than either CysLTs or PGE_2 in BAL of allergen-challenged mice. Spatial and temporal analyses during an acute inflammatory response indicate that maximal lipoxin levels recovered are delayed in onset compared with either LTs or PGs, and they are concurrent with resolution of inflammation, namely reduction of the exudate rather than initiation of inflammation[8]. Thus, the local levels of endogenously generated lipoxins after allergen challenge likely reflect not only the interval of sample acquisition, but also the cell origins of mediators present in BAL and other spatial relationships to cellular generators within lung.

LXA_4 interacts with ALX to mediate leukocyte-selective effects that promote resolution of inflammation[7,8]. Here, allergic pulmonary inflammation was markedly inhibited by expression of human ALX on murine leukocytes leading to decreases in total serum IgE, leukocyte tissue infiltration and cytokine and lipid-

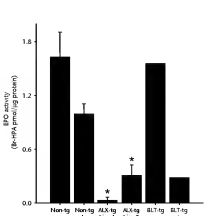

Fig. 6 Expression of human ALX in tg mice prevents eosinophil trafficking. LTB_4 (1 µg) and PGE_2 (1 µg) were topically applied (13–16 h) to mouse ear skin and EPO activity in tissue biopsies was determined by bromination of HPA. Values represent the mean ± s.e.m. for pmol Br-HPA per µg total protein for non-tg littermates ($n = 6$) or BLT-tg ($n = 2$) mice in the absence or presence of topical LXa (10 µg per ear) and 2 separate ALX-tg lines ($n = 4$). *, $P < 0.05$ by Student's *t*-test versus littermate controls.

mediator formation. Parallel decreases in bronchial hyper-responsiveness to methacholine were not observed in the ALX-tg mice. An uncoupling or dissociation of airway reactivity from inflammation *per se* has been reported in recent clinical trials[19,20], and the uncoupling here may have resulted from restricted expression of the hALX transgene to leukocytes rather than resident tissues of the airways. ALX-tg and non-tg mice given LXa had similar reductions in IL-13 and CysLTs. These findings suggest that the mechanisms for LXa-mediated inhibition of bronchial hyper-responsiveness following i.v. administration were distinct and likely secondary to the recently established direct interactions between LXa and recombinant CysLT1 receptors[12]. Taken together with the present findings these results also demonstrate that an increased ligand, as given pharmacologically via stable mimetic (LXa), or increased receptor (ALX) expression can prevent allergic pulmonary inflammation. Elucidation of endogenous regulators of lipoxin pathways may provide further insight into its role in inflammatory lung disease. Along these lines, whole blood from human aspirin-intolerant asthmatic individuals has reduced lipoxin biosynthetic capacity relative to aspirin-tolerant asthmatic or healthy individuals[35]. This may account, in part, for a more protracted and severe clinical course in patients with aspirin-intolerant asthma.

Our results are the first demonstration of direct protective and regulatory roles for lipoxin mimetics in airway hyper-responsiveness and asthmatic inflammation. In light of their ability to inhibit both of these key asthma phenotypes *in vivo*, lipoxin mimetics may represent a new treatment and therapeutic approach for asthma. Rather than inhibiting the actions of a single class of airway mediators to control asthma, lipoxin and their stable analogs could promote resolution of inflammation via multiple mechanisms, including inhibition of: 1) leukocyte (neutrophil[16], eosinophil and lymphocyte) recruitment and activation; 2) cytokine and chemokine production; 3) biosynthesis of pro-inflammatory lipid mediators; as well as 4) stimulating the non-phlogistic clearance of apoptotic leukocytes[34]; and 5) blocking edema formation[31]. Of note, lipoxin-stable analogs inhibit neutrophil responses *in vivo* with similar potency as corticosteroids[13]. Together, our results demonstrate the profile of *in vivo* actions of LXa and indicate that lipoxin mimetics and related compounds could provide novel therapeutic approaches to the treatment of airway hyper-responsiveness and pulmonary inflammation in select populations of human asthmatics.

Methods

Sensitization and challenge protocols. 5–7-wk-old male BALB/c (Charles River Laboratories, Wilmington, Massachusetts) or female hCD11b-hALX FvB transgenic (unpublished data) mice were housed in isolation cages under viral antibody-free conditions. After Harvard Medical Area IRB approval (Protocol #02570), mice were sensitized with intraperitoneal injections of OVA (Grade III; Sigma) (10 μg) plus 1 mg aluminum hydroxide (ALUM; J.T. Baker Chemical, Phillipsburg, New Jersey) as adjuvant in 0.2 ml PBS on days 0 and 7. On days 14–17, mice received 10 μg per mouse of LXa (ref. 16) (from D. Perez and J. Parkinson) or PBS with 1.6 mM $CaCl_2$ and 1.6 mM $MgCl_2$ (0.1 ml) by i.v. injection at least 1 h before aerosol challenge containing either PBS or 6% OVA for 25 min per day. The analog was designed to resist rapid enzymatic inactivation and was based on the structure of the aspirin-triggered 15-epi-LXA$_4$ (ref. 16), which carries its carbon-15 position alcohol in the *R* configuration or epimeric (*R*) to native LXA$_4$. On day 18, 24 h after the last aerosol challenge, airway responsiveness to i.v. methacholine (33–1000 μg/kg) was measured, bilateral BAL (2 aliquots of 1 ml PBS plus 0.6 mM EDTA) was performed and tissues were collected for histological analysis. Lung resistance (R_L) was measured using a sealed constant mass plethysmograph. The effective dose of methacholine required to increase R_L to 200% of control values was defined as the ED_{200} and used as an index of airway responsiveness[4,13]. For mice studied using a whole-body plethysmograph to assess airway responsiveness (Buxco, Sharon, Connecticut), each mouse was placed in a chamber and box pressure as a function of time was analyzed to yield the indicator of airflow obstruction, enhanced pause (Penh). PBS or methacholine (100 mg/ml) was given by aerosol through an inlet of the chamber for 4.5 min. Readings were initiated at 3 min and continued for 12 min. Peak and 1-min average Penh values were determined. Total serum IgE levels were determined by ELISA (Crystal Chem, Chicago, Illinois). *In situ* hybridization of murine ALX was performed using an anti-sense oligonucleotide probe (534 bp) corresponding to nucleic acids +581 to +1115 (GenBank Accession No. NM_008042) with the assistance of the Dana–Farber/Harvard Cancer Center Pathology Core Facility.

Allergen-initiated respiratory inflammation. Measurement of inflammatory mediators was determined in cell-free BAL fluid (2000*g*, 10 min) by sensitive and specific ELISA, in tandem, for IL-5, IL-13, eotaxin, TNF-α (R&D Systems, Minneapolis, Minnesota), LTB$_4$, cysteinyl LTs, PGE$_2$ (Cayman Chemical, Ann Arbor, Michigan), and LXA$_4$ (Neogen, Lexington, Kentucky). Cells were resuspended in HBSS, enumerated by hemocytometer, and concentrated onto microscope slides by cytocentrifuge (StatSpin, Norwood, Massachusetts) (265*g*). Cells were stained with a Wright–Giemsa stain (Sigma) to determine leukocyte differentials (after counting ≥ 200 cells).

Eosinophil tissue infiltration. 5 min after topical delivery of LXa (10 μg in 10 μl acetone) or vehicle control, PGE$_2$ (1 μg, Cayman) plus LTB$_4$ (1 μg, Cayman) were topically applied employing 10 μl acetone to the inside of ears of male mice (hCD11b-hALX FvB transgenic (unpublished data), hCD11b-hBLT FvB transgenic[36] or age- and gender-matched non-transgenic littermates; 4–5 months old; ~30 g body weight). After 13–16 h, 6-mm diameter skin punch biopsies (Acu-Punch, Fisher Scientific, Pittsburgh, Pennsylvania) were obtained. Samples were sliced finely with scalpels, homogenized in 400 μl potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, followed by three cycles of sonication and freeze-thaw and analyzed for eosinophil peroxidase (EPO) activity as described[36]. Briefly, trimethyl silylated HPA, Br-HPA and d_4-tyrosine were detected by GC-MS (GC model #6B90; MS model #5973; Hewlett Packard, San Fernando, California). HPA-TMS had a retention time of 7.01 min with diagnostic molecular ion (M^- = 310) and mass fragmentation, including *m/z* 295 [M^--CH_3], *m/z* 192 [M^--COOTMS], and a base peak of *m/z* 179 [M^--CH_3COOTMS]. Br-HPA-TMS had a retention time of 9.30 min with diagnostic molecular ion (M^- = 390) and mass fragmentation, including *m/z* 375 [M^--CH_3], *m/z* 272 [M^--COOTMS], and a base peak of *m/z* 259 [M^--CH_3COOTMS]. In addition, the mass spectrum of Br-HPA demonstrated the isotopic pattern of a monobrominated species. EPO activity was quantified by percent conversion of HPA to Br-HPA, taking into account the recovery of the internal standard (>80%) and normalized for the samples' protein content (determined by BioRad protein reagent) (BioRad, Hercules, California).

Acknowledgments

We thank M.H. Small for manuscript preparation, J. vom Saal for technical assistance and M. Lodo of the Dana Farber/Harvard Cancer Center Pathology Core Facility. G.T.D.S. was a recipient of a Partners Investigator Nesson Award. This study was supported, in part, by National Institutes of Health grants K08-HL03788 (to B.D.L.), P50-HL56383 (to J.M.D. and B.D.L.), HL-36110 (to G.T.D.S.) and GM-38765, DK-50305 and P01-DE13499 (to C.N.S.).

Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 23 MAY; ACCEPTED 11 JULY 2002

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